Limnol. Oceanogr., 44(2), 1999, 440–446

The nutrient stoichiometry of benthic microalgal growth: Redfield proportions are optimal

Abstract—Cellular nutrient ratios are often applied as indicators of nutrient limitation in phytoplankton studies, especially the so-called Redfield ratio. For periphyton, similar data are scarce. We investigated the changes in cellular C:N:P stoichiometry of benthic microalgae in response to different levels and types of nutrient limitation and a variety of abiotic conditions in laboratory experiments with natural inocula. C:N ratios increased with decreasing growth rate, irrespective of the limiting nutrient. At the highest growth rates, the C:N ratio ranged uniformly around 7.5. N:P ratios <13 indicated N limitation, while N:P ratios > 22 indicated P limitation. Under P limitation, the C:P ratios increased at low growth rate and varied around 130 at highest growth rates. For a medium with balanced supply of N and P, an optimal stoichiometric ratio of C:N:P = 119:17:1 could be deduced for benthic microalgae, which is slightly higher than the Redfield ratio (106:16:1) considered typical for optimally growing phytoplankton. The optimal ratio was stable against changes in abiotic conditions. In conclusion, cellular nutrient ratios are proposed as an indicator for nutrient status in periphyton.

The chemical composition of oceanic seston is known to be relatively constant at a C:N:P ratio of 106:16:1 (Redfield 1958; cf. Copin-Montegut and Copin-Montegut 1983). This biogeochemical ratio became widely known as the "Redfield ratio" and was subsequently physiologically interpreted for aquatic organisms. Droop (1974, 1975) investigated the nutrient content of phytoplankton within different limitation scenarios and developed the cell quota growth model. Internal nutrient ratios are equivalent to carbon-based cell quotas. Nitrogen and phosphorus supply supporting maximum growth rate was shown to lead to phytoplankton stoichiometry resembling the Redfield ratio (Goldman et al. 1979; Elrifi and Turpin 1985), and the internal nutrient ratios were proposed as an indicator of algal nutrient status (Healey and Hendzel 1980; Flynn 1990). Despite some criticism (Ryther and Dunstan 1971; Tett et al. 1985), biomass stoichiometry has been widely applied to assess nutrient supply to phytoplankton in marine (Paasche and Erga 1988; Burkhardt and Riebesell 1997) and freshwater studies (Sommer 1991*a*; Hecky et al. 1993). It should be noted that C:N:P ratios close to the Redfield ratio do not indicate the absence of light limitation (Tett et al. 1985). They just indicate that neither N nor P are limiting factors of growth (Goldman 1986).

The use of nutrient ratios as an index of nitrogen or phosphorus limitation was also recommended for benthic microalgae (Borchardt 1996), because of the close phylogenetic relationship between benthic and pelagic microalgae. However, in benthic studies, it has seldom been applied to date (Engle and Melack 1993; Rosemond 1993; Rosemond et al. 1993; Hillebrand and Sommer 1997). The infrequent use may be because the relationship between cellular stoichiometry and growth rate for marine microphytobenthos has not yet been tested experimentally. Kahlert (1998) recently reviewed literature data from freshwater periphyton and found that C:N:P ratios are a reliable tool for the assessment of the nutrient status of benthic algae, proposing an optimum ratio of 158:18:1.

In the present study, we wanted to answer the following questions: (1) Is there a consistent relationship between benthic microalgal growth rates and cellular stoichiometry, and (2) Is this relation independent of abiotic conditions?

To investigate the response of internal nutrient ratios to changes in nutrient regimes, we used a semicontinuous dilution of culture media combined with sampling in intervals. We used natural inocula of algae to simplify comparison to natural assemblages. The algae for the inocula were scraped from an artificial substrate in the Kiel Fjord, Western Baltic Sea, 10 d before the experiments were started. The algae were cultured in unenriched filtered seawater under the same abiotic conditions as the experimental treatments (Table 1). At the beginning of the experiments, 1 ml of the inoculum was added to each treatment. The experiments were conducted in flat-bottom, transparent, polystyrene culture flasks with 30 ml total medium content. The algae grew as a biofilm on the bottom of the flasks, which were shaken once daily. This biofilm was a dense monolayer of cells lacking an overstory. It can therefore be assumed that CO_2 was available in excess.

The media used in the experiments consisted of organism-free–filtered seawater (0.2-μm cellulose-acetate filters) from the same location, enriched with nutrients and trace metals. The balanced medium (designated V) contained 80 μmol liter^{-1} N (as $NaNO_3$), 80 μmol liter^{-1} Si (as $Na_2O_3Si \times 5H_2O$), and 5 μmol liter^{-1} P (as $Na_2HPO_4 \times 2H_2O$), resulting in a medium N:P ratio of 16. For the N-limited medium (designated N_{lim}), the nitrogen concentration was reduced to 10 μmol liter^{-1}. For the Si-limited (Si_{lim}) media, Si was reduced to 10 μmol liter^{-1}; for the P-limited medium (P_{lim}), no phosphate was added, resulting in 1.0 μmol liter^{-1} P. The experiments were conducted in autumn 1997 and spring 1998. In the autumn experiment, four different media were applied, and the treatments consisted of an alteration of dilution rate (Table 1). In spring 1998, three different media were used, and the temperature was altered (Table 1). The two experiments differed furthermore in the taxonomic composition of the inoculum and in the light intensity, which was measured with a LiCor LI 189 (Table 1). Each treatment was conducted in triplicate, resulting in 24 cultures in autumn (four media × two dilution rates × three replicates) and 27 cultures in spring (three media × three temperatures × three replicates).

Thrice a week, the algae were counted alive employing an inverted microscope (Leitz DMIRB) at ×630 magnification. Up to 400 cells were counted per sample. To compare

Table 1. Treatments in limitation experiments with natural algal inocula. The table lists the code of the experiment, the type of dilution rate, the duration of the experiment, the light intensity, the temperature, and the media used.

Code	Dilution rate (d^{-1})	Time	Light (μmol m^{-2} s^{-1})	Temp. (°C)	Applied media
H (high dilution)	0.5	22 Sep–10 Nov 97	21	14	V, N_{lim}, P_{lim}, Si_{lim}
L (low dilution)	0.07	Ditto	21	14	V, N_{lim}, P_{lim}, Si_{lim}
C (cold temp.)	0.5	19 Jan–9 Mar 98	35, 8	5	V, N_{lim}, P_{lim}
M (medium temp.)	0.5	Ditto	34, 7	14	V, N_{lim}, P_{lim}
W (warm temp.)	0.5	Ditto	32, 2	19	V, N_{lim}, P_{lim}

the different species, which span several size classes, biovolume was calculated by fitting appropriate geometric models (Hillebrand et al. submitted). In addition, thrice a week, a sample was taken with a sterile pipette from the bottom of the flask. The sample volume corresponded to the daily dilution, i.e., 15 ml for treatments C, M, W, and H (for codes, *see Table 1*) and 5 ml for treatment L. The samples were divided and filtered on precombusted Whatman GF/C filters for analyses of particulate CN and P, respectively. Particulate phosphate was determined as orthophosphate after a combined digestion with heat and acid (Hillebrand and Sommer 1997). Since this analysis needs a high amount of material, the three replicates of one treatment had to be pooled. Particulate carbon and nitrogen were measured with a Fisons CN-Analyzer (NA 1500N).

The experiments were divided into two phases. After 28 d, the daily medium dilution was stopped to enforce a stronger limitation by decreasing the supply with new media. Sampling was carried out once a week. After sampling, the total volume of the cultures was filled up to 30 ml again. The L treatment was stopped at day 28, but the other experiments were conducted until day 49.

The daily growth rates μ were calculated from the total biovolume of each replicate according to Eq. 1:

$$\mu = \frac{\ln B_2 - \ln B_1}{t_2 - t_1} \quad (1)$$

including

B = biovolume.

t = time.

The ratios of C:N, N:P, and C:P, respectively, were calculated on a molar basis. They were compared to the positive daily growth rates by a three-parameter exponential equation (Eq. 2). The use of ratios in regression analysis is not with-

Table 2. Fit of exponential function (Eq. 2) to C:N, C:P, or N:P ratio dependent on μ. The table lists treatments and media, ratio for which the regression was calculated, number of data, coefficient of determination r^2, and parameter estimates, as well as the F-ratio between regression explained mean squares and residual mean square (Sokal and Rohlf 1995). Significance level: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, not significant. n.r., no regression (estimation procedure did not converge).

Treatment	Med	Ratio	n	r^2	a	b	c	$F_{(2;n-3)}$
All	All	C:N	322	0.1669	7.5 ± 0.7	1.8 ± 0.1	−6.8 ± 2.6	32.04***
All	V	C:N	102	0.3692	7.2 ± 0.6	1.9 ± 0.1	−9.9 ± 4.2	28.97***
All	P_{lim}	C:N	98	0.362	5.9 ± 0.9	1.8 ± 0.2	−5.0 ± 2.3	26.96***
All	N_{lim}	C:N	96	0.1118	7.5 ± 2.6	2.0 ± 0.2	−3.1 ± 2.5	5.78**
All	Sl_{lim}	C:N	26	0.1952	8.5 ± 0.6	1.8 ± 0.6	−27.5 ± 18.3	10.18**
C	All	C:N	77	0.3847	4.4 ± 2.4	2.2 ± 0.2	−3.3 ± 1.9	23.14***
M	All	C:N	63	0.2253	6.8 ± 2.3	2.3 ± 0.3	−6.0 ± 4.3	7.99**
W	All	C:N	63	0.2675	7.6 ± 0.8	2.6 ± 0.3	−26.2 ± 11.2	10.96**
H	All	C:N	89	0.1796	8.4 ± 0.4	1.3 ± 0.2	−12.1 ± 7.3	9.42***
L	All	C:N	30	n.r.	—	—	—	—
All	All	C:P	122	0.0655	143.6 ± 48.5	5.1 ± 0.3	−8.2 ± 10.6	4.17*
All	V	C:P	39	0.2380	119.0 ± 50.7	5.1 ± 0.4	−5.1 ± 4.2	3.28*
All	P_{lim}	C:P	37	0.4306	139.2 ± 47.8	6.1 ± 0.2	−17.5 ± 8.0	12.85**
All	N_{lim}	C:P	36	n.r.	—	—	—	—
All	Sl_{lim}	C:P	10	0.4067	51.2 ± 53.8	4.9 ± 0.5	−8.6 ± 10.4	2.40 ns
C	All	C:P	23	0.2076	80.3 ± 61.9	5.0 ± 0.5	−5.8 ± 6.9	3.54 ns
M	All	C:P	20	0.2618	73.3 ± 120.4	5.7 ± 0.4	−5.4 ± 6.0	2.23 ns
W	All	C:P	21	0.1655	116.7 ± 27.4	4.6 ± 0.6	−20.7 ± 22.0	1.79 ns
H	All	C:P	26	n.r.	—	—	—	—
L	All	C:P	18	0.1366	216.1 ± 105.0	5.8 ± 0.7	−15.6 ± 15.0	1.17 ns
All	P_{lim}	N:P	37	0.2668	20.9 ± 2.6	3.1 ± 0.3	−24.7 ± 14.6	6.50**

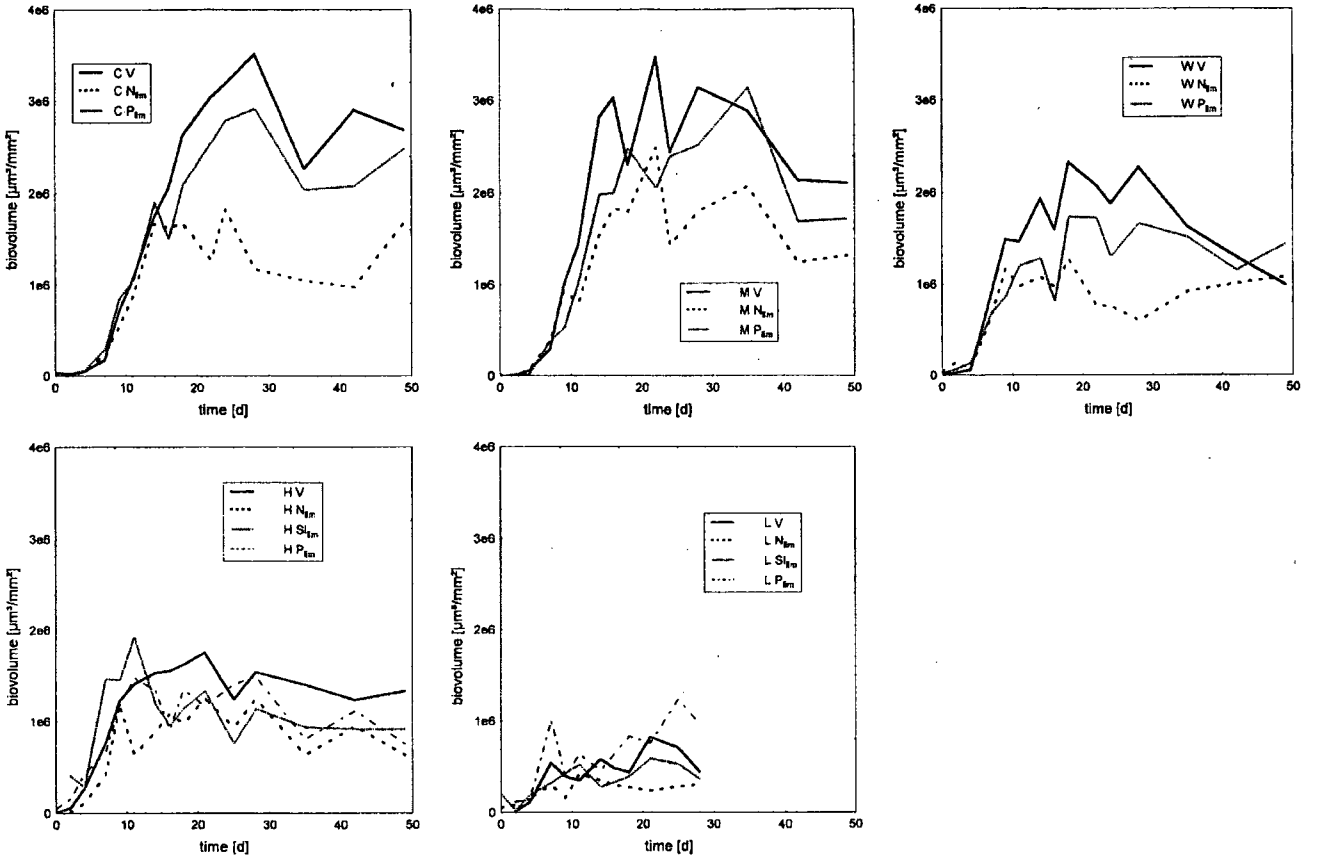

Fig. 1. Development of mean of total biovolume ($\mu m^3/mm^2$) of benthic microalgae over time for all treatments and media of experiments from autumn and spring.

out difficulties (Sokal and Rohlf 1995), but normal distribution of the data of all ratio variables was affirmed ($P > 0.05$, Kolmogorov-Smirnov test, Statistica 5.1).

$$R = a + e^{(b+c*\mu)} \quad (2)$$

with

R = molar ratio of C:N, N:P, and C:P, respectively.

μ = daily growth rate.

a, b, c = parameters.

With a negative estimate for c, the resulting curve decreases and approaches asymptotically to a horizontal line with $y = a$. Therefore, parameter a can be taken as an estimate of the optimal ratio. The validity of the regression model was estimated by an analysis of variance comparing the model-explained variance with the residual variance (Sokal and Rohlf 1995). The presence of a global convergence minimum in the regression procedure was affirmed by using different software and different estimation procedures (Statistica 5.1 and Statgraphics 6.1).

Total biovolume of the treatments followed a sigmoidal curve in all cases, explaining 87.03% ± 12.62 (mean ± SD) of the variance (Fig. 1). Final biovolumes were generally higher at lower temperature and lower in the N_{lim} treatments. Moreover, the spring experiments resulted in higher final biovolume than the autumn experiment; the latter again was divided in lower biovolume in L treatments compared to H treatments (Fig. 1). Daily growth rates increased in the beginning of the experiment and decreased afterwards, varying around zero after attainment of the carrying capacity (Fig. 2). In the autumn experiments, 30 species were present, representing the Bacillariophyceae, Chlorophyceae, and cyanobacteria. In spring, 30 species represented the Bacillariophyceae, cyanobacteria, and Rhodophyceae. Almost all treatments were dominated by diatoms (unicellular, chain-forming, and tube-dwelling), whereas nonheterocystous cyanobacteria became codominant at 19°C temperatures and high N content.

C:N ratios increased with time (significant regression slopes over time, $P < 0.05$, except for LV, LN_{lim}, and HSI_{lim}), up to 20 in P_{lim}, up to 23 in V, and up to 45 in N_{lim} cultures, respectively. This increase reflected the increasing strength of nutrient limitation. The ratio of C:N decreased exponentially with increasing growth rate (Fig. 3; Table 2), independent of treatment and limiting nutrient. The regression model described the relationship between C:N ratios and growth rates significantly (F-ratio, $P < 0.01$; Table 2) for all media and treatments, except treatment L. The optimal ratio at high growth rates, estimated by parameter a of the regression, is

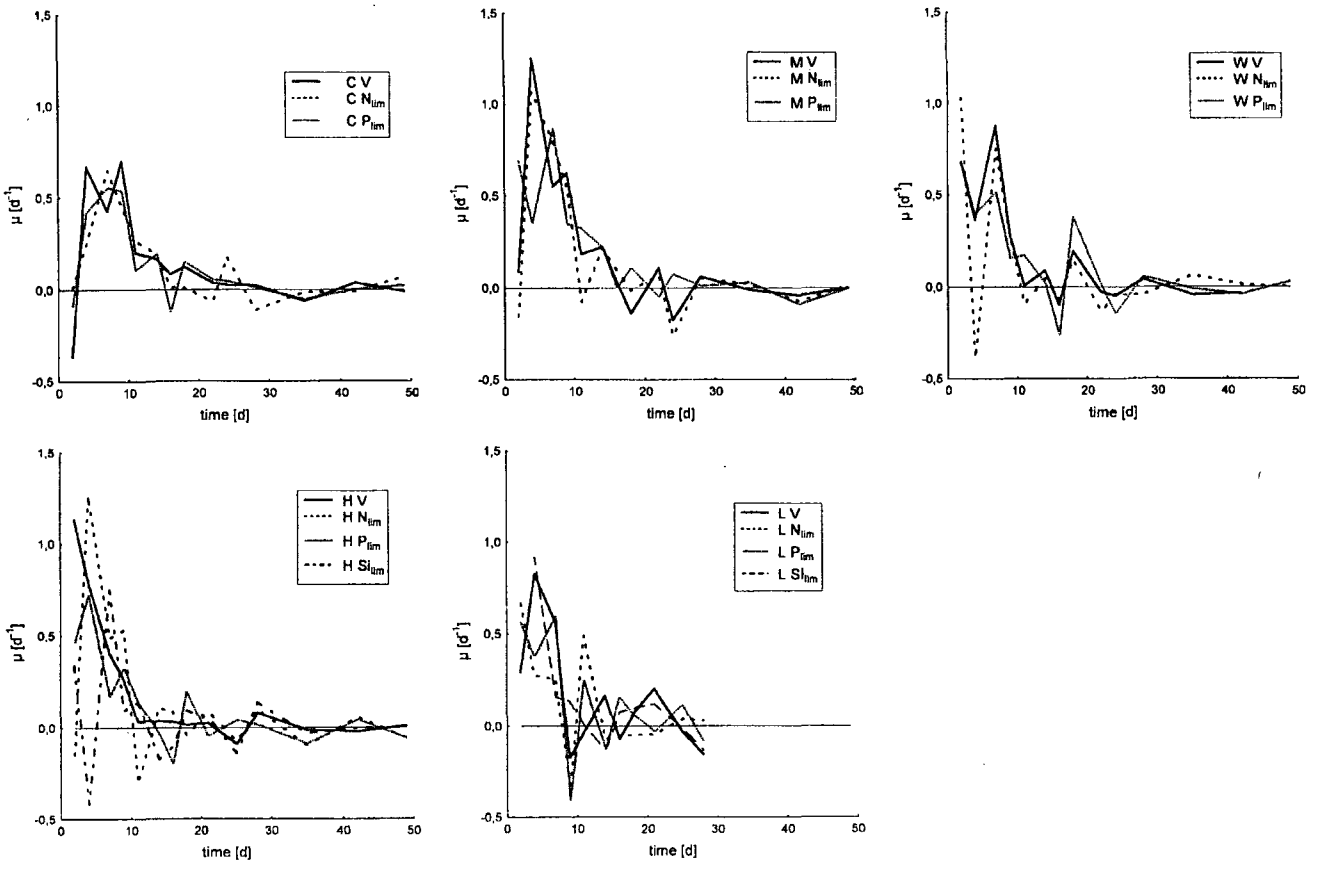

Fig. 2. Mean of daily growth rate (Eq. 1) in relation to time.

plotted for the different treatments and media in Fig. 4, compared to their standard error (except for the L treatment). The estimates of the optimal C:N ratios showed widely overlapping confidence intervals (Fig. 4). A difference between the optimal C:N ratios of different treatments could therefore not be sustained; thus, C:N ratios increased irrespective of the limiting nutrient.

The three-parameter exponential regression on C:P data was significant at $P < 0.01$, only for the P_{lim} media, and at $P < 0.05$ also for V and the pooled data (Table 2). In an Si- or N-limited situation, there was no consistent relation between C:P ratios and growth rate. Only under P_{lim} conditions did the N:P ratio increase exponentially with decreasing growth rate (Table 2). N:P ratios in the stationary phase ($\mu < 0.1$) were significantly different when comparing different media ($P < 0.05$; Kruskal-Wallis analysis of variance [ANOVA] on ranks; all pairwise multiple comparison by Dunn's method, Sigma Stat) but not between different abiotic conditions (Fig. 5). The mean of the N:P ratio was significantly lower at N_{lim} (10.69) compared to P_{lim} (33.62), while the cellular N:P was intermediate at balanced medium N:P (for V 20.58, for Si_{lim} 14.22).

Conclusively, these results show that high C:N ratios were due to nutrient limitation in general, while C:P and N:P ratios increased significantly only with P limitation.

Our data support the view that biomass stoichiometry is an indicator of nutrient status for benthic microalgae as for phytoplankton, for which it was developed experimentally and conceptually (Droop 1974, 1975; Healey 1978; Healey and Hendzel 1980). Similar to our results, the C:P and N:P ratios of pelagic microalgae increased with decreasing growth rate under P limitation, while the C:N ratio increased with decreasing growth rate under P and N limitation. Moreover, the increase of C:N at low growth rates was higher under N limitation than under P limitation in our data and in phytoplankton studies (Perry 1976; Sakshaug and Holm-Hansen 1977; Goldman et al. 1979; Healey and Hendzel 1980). Under N limitation, the C:P and N:P ratios were found to decrease with decreasing growth rates (Elrifi and Turpin 1985). It can be concluded from these data that C:N ratios are applicable as general indicators of limitation, while C:P and N:P ratios allow indication of P vs. N limitation.

The criticism concerning the indicator value of the nutrient ratios pointed mainly at light-limited conditions (Tett et al. 1985; Wynne and Rhee 1986), leading to the conclusion that light limitation was not reflected by biomass stoichiometry. In agreement with Goldman (1986), the optimal ratios emerging from our autumn experiment with lower light intensities did not differ from the ratios from the spring experiment (Fig. 4). This contradicts Wynne and Rhee (1986), who described changes in optimum N:P ratios in planktonic algae caused by changes in light intensity and wavelength.

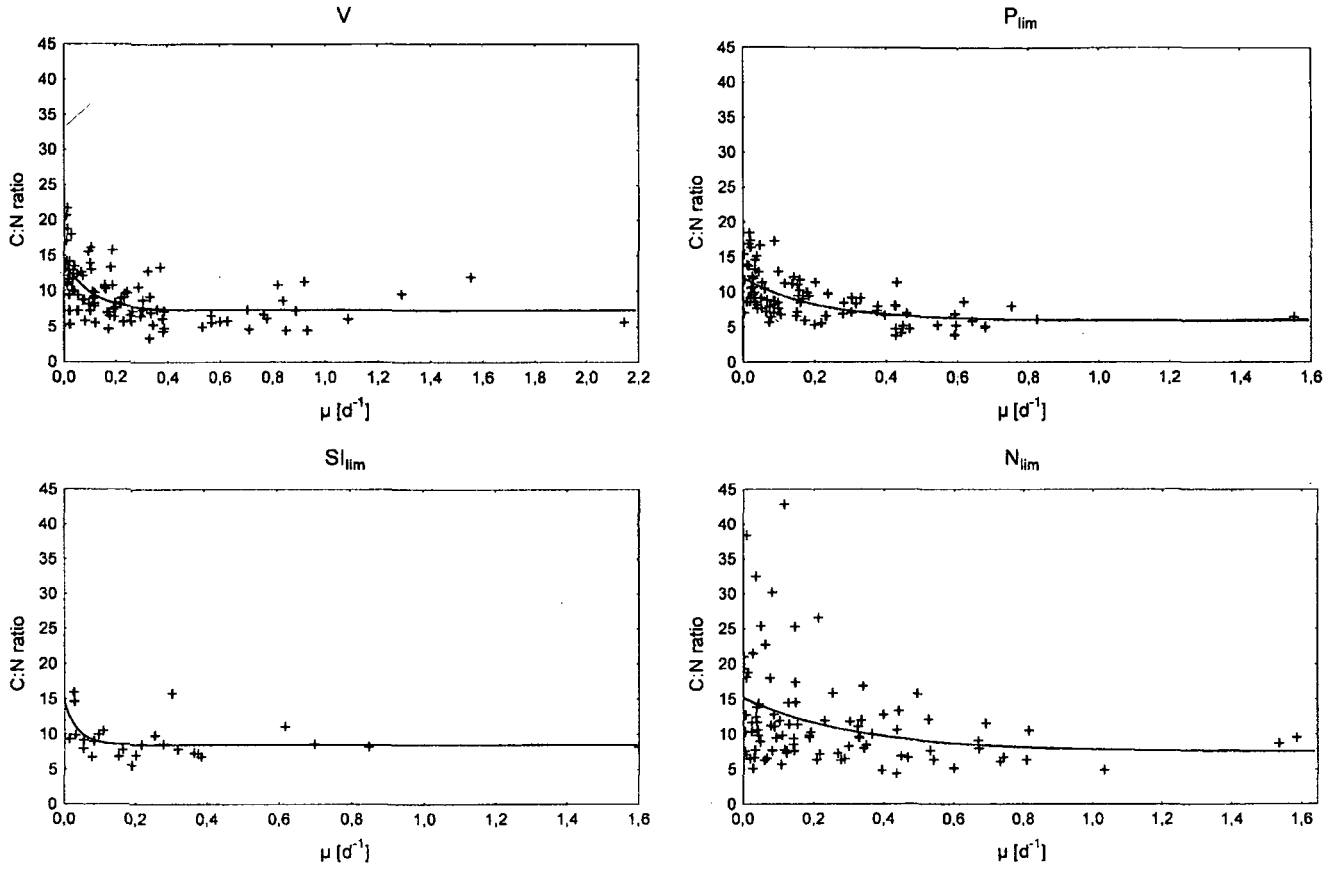

Fig. 3. C:N ratios of benthic microalgae dependent on growth rate, plotted for different media. Lines represent nonlinear fits of Eq. 2. Parameter estimates are presented in Table 2.

However, they calculated the optimum N:P ratio from minimal cell quotas, which refer to the stationary (i.e., limited) phase of cultures ($\mu = 0$). This is a contradiction to the concept of optimal ratios ($\mu = \mu_{max}$), as becomes evident, if we approach our data in the same way. The minimal cell quotas (q_0) of N and P can be calculated from Eq. 2 by substituting $\mu = 0$ and inserting the estimates of a and b from Table 2. The mean ratio of q_0N:q_0P was 18.56 for all treatments, whereas the ratio for P_{lim} was 48.8. The latter ratio reflects the nutrient-limited situation and not an optimal ratio. Besides, these calculations show that cell quotas of benthic microalgae (ranges of q_0 are 0.060–0.083 mol N mol^{-1}C and 0.002–0.008 mol P mol^{-1}C, respectively) are within the same order of magnitude as those reported from freshwater phytoplankton (Sommer 1991*a,b*).

Based on the work of Healey and Hendzel (1980), Hecky et al. (1993) suggested limits of indicating values for phytoplankton nutrient ratios. Moderate N limitation was indicated by C:N > 8.3 and severe limitation > 14.6, whereas moderate P limitation was indicated by C:P > 129 and severe limitation by C:P > 258 and N:P > 22.

From our data, an optimal ratio of fast-growing periphyton can be determined in analogy to the optimal phytoplankton ratios, based on the estimates of a (Eq. 2) for the balanced experiments (medium V). This results in a C:N:P ratio of 119:17:1. However, it seems to be more useful to give ranges of ratios in nonlimited conditions. These ranges were calculated from the estimates for the different media (V, P_{lim}, N_{lim}, and Si_{lim}; Table 2), adding the standard error to the maximum estimate and subtracting it from the minimum. The optimum ranges are 5–10 for C:N and 90–185 for C:P. N:P ratios between 13 and 22 indicate balance between nitrogen and phosphate. These ranges allow the establishment of C:N:P ratios as indicators of nutrient limitation (Fig. 6). With an N:P ratio < 13 and a C:N ratio > 10, the periphyton can be assigned N limited. With an N:P ratio > 22 and a C:P ratio > 180, the microbenthic assemblage is P limited.

These ratios are more similar to the Redfield ratio and the limits given by Hecky et al. (1993) for phytoplankton than to the optimal ratio proposed for freshwater periphyton by Kahlert (1998). Based on a literature survey, she found an optimum ratio for freshwater periphyton slightly higher than the Redfield ratio (C:N:P = 158:18:1) and proposed much higher indicators of limitation than Hecky et al. (1993): C:P > 369 and N:P > 32 for P limitation and C:N > 11 and N:P < 12 for N limitation. The reason for the discrepancy is probably because macrophytes (e.g., *Cladophora*) were included in the data survey, which are known to differ in their C:N:P ratios. Atkinson and Smith (1983) found a median of C:N:P of 550:30:1 in a comparison of marine

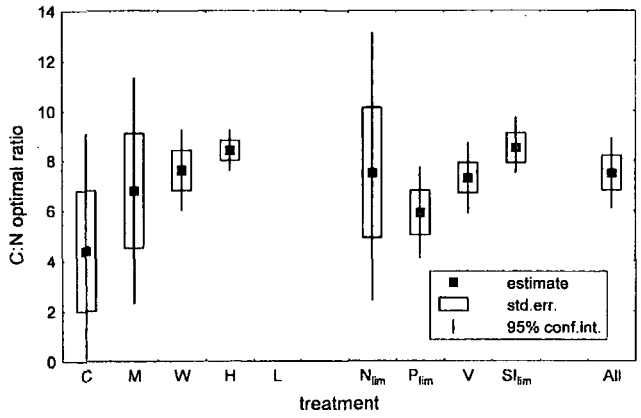

Fig. 4. Optimal C:N ratios of different treatments (M–L) and media (N_{lim}–Si_{lim}). Optimal C:N ratios are presented as estimate of parameter a (Eq. 2), its standard error, and confidence interval. For treatment L, no optimal ratio could be fitted. "All" represents the optimal ratio fitted on all data.

macroalgae, which are supposed to contain more structural carbon. In their data, however, the Redfield ratio posed a lower limit of the found ratios, which may be reached only in very nutrient-rich localities like Antarctic waters (Weykam et al. 1996). C:N:P ratios were reported from studies dealing with microphytobenthos before, but they were reported without relating them to growth rates. The ratios were in the range reported here (Reuter et al. 1986; Engle and Melack 1993; Rosemond 1993; Rosemond et al. 1993; Turner et al. 1994; Vymazal and Richardson 1995; Hillebrand and Sommer 1997). Therefore, the limits for nutrient ratios proposed by us can be applied for periphyton dominated by diatoms and cyanobacteria.

A note has to be added in proof: Biomass stoichiometry of natural benthic communities may be influenced by a high proportion of detritus or by carbon limitation, both of which were excluded in our experiments. The influence of detritus was found to be less than expected for phytoplankton (Healey and Hendzel 1980), but it has to be evaluated for periphyton (cf.

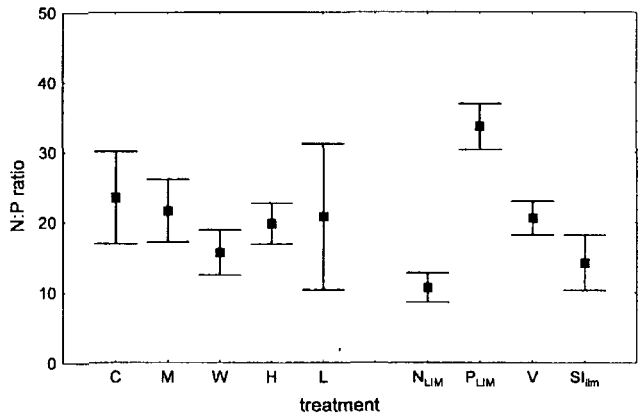

Fig. 5. N:P ratios of stagnant cultures of different treatments and nutrient content, depicted as mean ± SE of all sample dates with $\mu < 0.1$.

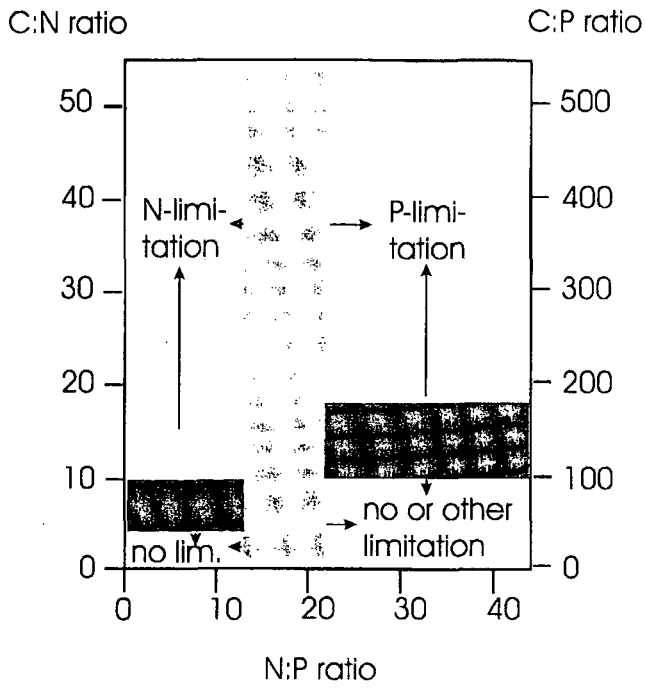

Fig. 6. Schematic diagram on the use of nutrient ratios as indicator of nitrogen or phosphorus limitation. Shaded bars represent the range of optimal ratios. Further explanation is given in the text.

Kahlert 1998). Carbon limitation was shown to change C:N:P ratios in a marine pelagic diatom, by decreasing C:P and N:P ratios and increasing C:N ratios (Burkhardt and Riebesell 1997). Furthermore, it should be noted that in dense biofilms, the algae could have different access to nutrients. This depends on whether these stem from the water column or from sediment pore water, so that emerging nutrient stoichiometry may be the mean of a gradient.

In conclusion, cellular nutrient ratios are a useful approach for the detection of nitrogen or phosphorus limitation in benthic microalgae as well as in phytoplankton. The following constraints should be observed: The cellular C:P ratio is an index of P limitation, the cellular C:N ratio indicates limitation in general, and the N:P ratio distinguishes between N or P limitation.

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Acknowledgments

This study was initiated by discussions with Maria Kahlert, University of Uppsala. Financial support was granted from the Institut für Meereskunde and the Deutsche Forschungsgemeinschaft (grant SO145/15-1). The help of Thomas Hansen during the CN analysis is gratefully acknowledged. The manuscript profited from comments by Monika Feiling, Ulrike G. Berninger, Rolf Karez, Carlos M. Duarte, Stephen V. Smith, and one anonymous reviewer.

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Received: 9 April 1998
Accepted: 6 November 1998